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Hepatic hazard assessment of silver nanoparticle exposure in healthy and chronically alcohol fed mice

Ali Kermanizadeh*, Nicklas R. Jacobsen[†], Martin Roursgaard*, Steffen Loft* and Peter Møller*

*University of Copenhagen, Department of Public Health, Section of Environmental Health, Copenhagen, Denmark

[†]National Research Centre for the Working Environment, Copenhagen, Denmark

Corresponding author:

Ali Kermanizadeh,

University of Copenhagen, Department of Public Health, Section of Environmental Health, Øster Farimagsgade 5A, Copenhagen, 1014, Denmark

alke@sund.ku.dk

Tel no: +44 7376133108

Abstract

Silver (Ag) nanoparticles (NPs) are currently among one of the most widely used nanomaterials. This in turn, implies an increased risk of human and environmental exposure. Alcohol abuse is a global issue with millions of people in the general population affected by the associated adverse effects. The excessive consumption of alcohol is a prominent cause of chronic liver disease which manifest in multiple disorders. In this study the adverse health effects of Ag NP exposure were investigated in models of alcoholic hepatic disease *in vitro* and *in vivo*. The data showed that Ag NP induced hepatic health effects were aggravated in the alcohol pre-treated mice in comparison to controls with regards to an organ specific inflammatory response, changes in blood biochemistry, acute phase response and hepatic pathology. In addition, alcoholic disease influenced the organ's ability for recovery post NP challenge. Additionally, it is demonstrated that the *in vivo* data correlated well with *in vitro* findings where ethanol pre-treatment of hepatocytes resulted in significantly increased inflammatory response post Ag NP exposure. To the best of our knowledge this is the first study of its kind to investigate nano-sized material-induced hepatic pathology in models representative of susceptible individuals (those with pre-existing alcohol liver disease) within the population. This is an area of research in the field of nanotoxicology, and in particular with regards to NP risk assessment that is almost entirely overlooked.

Keywords: Liver, alcohol abuse, Ag NPs, inflammation, acute phase response, pathology

Introduction

The rapid expansion of the commercial use of atomic or molecular scale materials has led to an escalating interest in the fields of nanoscience, nanotechnology and nanomedicine (Bhattacharya et al. 2012). The unique properties of engineered nanoparticles (NPs) (i.e. size, surface charge, composition, shape, solubility, etc.) which make them desirable to numerous industries might also contribute to their potential toxicity (Johnston et al. 2012). The probability for public and occupational exposure is likely to rise with increasing production and utilisation of NPs; therefore, there is an urgent need to consider the possibility of health consequences of manufactured NP exposure. The risk assessment for nanomaterials (NMs) to human health heavily relies on the appropriate use of dose-response relationships of adverse effects, the understanding and the correct use of appropriate routes of exposure, a thorough understanding of the potential for translocation, distribution, accumulation and bio-persistence of materials as well as the importance of the utilisation of the relevant models for toxicological testing.

Silver (Ag) NPs are widely utilised as an additive in various textiles and plastics due to their anti-microbial properties. They are also heavily used for treatment of wounds and burns, as well as being utilised in water disinfection, as health supplements, in food preservation and numerous health care devices (Ahamed et al. 2010). Therefore, these particles are ingested by humans and reach the gastrointestinal tract (GIT). Once in the GIT there is potential for particle uptake by Peyer's patches or enterocytes (Geiser et al. 2010; Kermanizadeh et al. 2015). It has been suggested that, once in the sub-mucosal tissue, NPs are able to enter the lymphatics and the bloodstream. Additionally, it is widely demonstrated that the liver has high significance with regards to nano-sized material accumulation and toxicity following intravenous (IV) exposure compared to other organs (Lipka et al. 2010; Sadauskas et al. 2009) and alongside the kidneys might be responsible for the clearance of NMs from the blood (Geiser et al. 2010; Semmler-Behnke et al. 2008). In addition, advances in the field of nanomedicine has resulted in an increase in direct entry of NPs into the circulatory system (NPs may be deliberately introduced into the body by injection) (Walczyk et al.

2010). The presence of these NPs in the blood allows distribution to a wide range of target organs, increasing the likelihood of NP accumulation in the liver with potential adverse effects (Geraets et al. 2014).

The liver is the metabolic centre of the body and has a crucial role in maintaining systemic homeostasis. It is responsible for the storage, synthesis, metabolism and redistribution of carbohydrates, fats and vitamins (Kmiec 2001). The liver produces large numbers of serum proteins and an array of enzymes. It is also the principal detoxification centre of the body, removing xenobiotics and waste products by metabolism or biliary excretion (Kmiec 2001).

Alcohol abuse is a global issue and contributes to more than 200 diseases and causal factor for an estimated 4 million deaths annually (World Health Organisation 2014). The excessive consumption of alcohol is a prominent cause of chronic liver disease which manifest in multiple disorders from fatty liver (steatosis) to more severe forms of liver injury such as alcoholic hepatitis, cirrhosis and hepatocellular carcinoma (Rocco et al. 2014). As an additional complication, there is a large variation in the total alcohol consumption by individuals in the general public and even a wider spectrum of adverse effect associated with alcohol abuse, indicating that a significant proportion of the overall population could be suffering from sub-clinical symptoms associated with alcohol abuse without any apparent visible disease manifestations. The liver is the predominant organ for ethanol metabolism; and is therefore a major target of adverse effects caused by the consumption of alcohol (Bertola et al. 2013).

In the present study, a wide array of *in vitro* and *in vivo* biomarkers linked to liver disease (inflammation, alterations in blood parameters, anti-oxidant depletion, acute phase response and pathology) were investigated following treatment with a Ag NP both in healthy and alcohol abuse disease models. To the best of our knowledge this is the first study of its kind to investigate nano-sized material-induced hepatic effects in models representative of individuals with pre-existing medical conditions caused by misuse of alcohol. This is an area of research in the field of nanotoxicology and in particular with regards to NP risk assessment that is almost entirely overlooked.

Materials and methods

Nanoparticles

BioPure 10 nm citrate-coated Ag NPs (NanoComposix, Czech Republic) were supplied in a concentration of 1 mg/ml in a 2 mM sodium citrate buffer. According to the manufacturer the ionic Ag in the sample was less than 0.02%.

Characterisation of the Ag NPs

The morphology, particle size and the surface charge of the Ag NPs has been previously reported (Guo et al. 2016). The hydrodynamic size distributions of the NPs dispersed in PBS and hepatocyte complete medium was determined in the 10-50 µg/ml concentration range by Nanoparticle Tracking Analysis (Nanosight LM20, UK). A limulus amoebocyte lysate (LAL) Pyrogen™ Plus assay (Lonza, Switzerland) was utilised to test for possible endotoxin contaminations of the Ag NPs. The kit was used according to the manufacturer's guidelines. The NPs were vortexed for 15 seconds just prior to dilution in PBS for the *in vivo* experiments or complete hepatocyte cell culture medium for the *in vitro* studies.

In vitro cell culture ethanol and particle treatment

The human hepatocellular carcinoma cell line (HepG2) (Sigma Aldrich, UK) was maintained in Minimum Essential Medium Eagle (MEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml Penicillin/Streptomycin and 1% non-essential amino acids (all Sigma Aldrich, UK). On occasion and when required the HepG2 cells were exposed to 50 mM of ethanol for 24 hr. The hepatocytes (10⁴ cells per well (96 well plates - TRP, Switzerland) in 100 µl of the cell culture medium) were exposed to the Ag NPs in a concentration range between 0.62-39 µg/cm² (equivalent to 2-125 µg/ml) for a further period of 24 hr. The highest concentration corresponds to the IV injected dose of 100 µg/mouse.

WST-1 cell viability assay

After the material exposure, the plates were washed twice with PBS, followed by the addition of 10 µl of the WST-1 cell proliferation reagent (Roche, USA) and 90 µl of fresh medium before incubation for 1 hr at 37°C. The supernatant was transferred to a fresh plate and the absorbance measured by dual wavelength spectrophotometry at 450 and 630 nm using a micro-plate reader (supernatants were transferred into fresh plates to decrease the potential interference of the materials during measurements).

HepG2 cell interleukin (IL) 8 production

After a 24 hr NP exposure, the HepG2 supernatants were collected and stored at -80°C and later used for enzyme linked immunosorbent assays (ELISA). The supernatants were centrifuged at 1000 g and the IL8 levels determined by ELISA according to the manufacturer's instructions (human IL8, ELISA kit - Life Technologies, USA).

Animals, diets and Ag NPs treatments

8-week-old female C57BL/6N mice were obtained from Taconic, Denmark. The mice were housed in polypropylene cages with bedding (sawdust) at controlled environmental conditions as previously described (Kyjovska et al. 2015). The mice were allowed to acclimatize for a period of 5 days (regular diet of mouse chow; Altromin 1324) before being transferred to an all liquid diet (Lieber-DeCarli 82 rodent liquid diet, control, Bio-Serv, USA) for a further 5 days. At this juncture the animals were divided into two groups with one receiving the same liquid diet for 25 days while the other group were fed an all liquid diet supplemented with 5% ethanol (Lieber-DeCarli 82 rodent liquid diet, ethanol, Bio-Serv, USA) (Figure 1). A study on potential adverse effects of liposome nanocarriers was carried out in parallel with similar study design and is to be reported elsewhere.

FIG 1

The mice were housed in groups of 5 with each animal being offered 20 ml of the liquid diet per day (animals fed every morning at the same time). The nutritional information of both diets is provided in Table 1. The access to water was provided *ad libitum*. The mice were weighed every 5 days during the feeding procedure as well as on the day on which they were sacrificed. Following the feeding period, the animals were injected via the lateral tail vein with 25, 100 µg of Ag NPs, 158 µg of silver nitrate (AgNO₃) (as the ionic control molecular weight - equivalent to 100 µg of Ag per animal) or PBS (a volume of 100 µl for all treatments) (Table 2). The mice were kept under 3.5% isoflurane anaesthesia during the injection process. Following a 24 or 168 hr exposure period the mice were sacrificed by exsanguination while anaesthetized (200 µl of ZRF cocktail composed of Zoletil 250 mg/ml, Rompun 20 mg/ml and Fentanyl 50 µg/ml) after collection of intracardial blood. The liver was removed; with the caudate lobe (glutathione measurements) and the left lateral lobe (inflammation) snap frozen in liquid nitrogen and stored at -80°C, while the right medial lobe was fixed in a 4% formaldehyde solution for histological analysis. C57BL/6 mice were chosen as they are the preferred strain to use for *ad libitum* ethanol feeding. Some other mice strains can either resist eating the diet with high ethanol concentrations or are adversely affected by higher alcohol-containing diet (Bachmanov *et al.* 1996; Bertola *et al.* 2013).

All animal experiments were conducted under the Danish federal guidelines for use and care of laboratory animals (complied with the EC Directive 86/609/EEC) and approved by the Danish animal inspectorate (licence number: 2012-15-2934-00223).

TABLE 1

TABLE 2

Analysis of blood biomarkers relating to liver toxicity

Intracardial blood samples were centrifuged and serum was frozen at -80°C for the analysis of biomarkers and the acute phase response. The clinical chemistry measurements included aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, triglycerides and albumin. The samples were thawed, centrifuged for 10 min at 2000 g at room temperature with the analysis of the supernatant performed on a Cobas 8000 modular analyser (Roche, USA).

Acute phase response

The acute phase protein serum amyloid A3 (SAA3) was measured in the serum from the Ag NP exposed animals and the controls utilising a commercially available mouse SAA3 ELISA kit (Merck Millipore, Denmark) according to the manufacturer's instructions.

Inflammatory response

The liver samples were all thawed on ice, weighed and homogenised in a homogenisation buffer (PBS containing 1% Triton X-100 (Sigma, UK) a protease inhibitor cocktail (104 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 80 µM of Aprotinin, 4mM Bestatin, 1.4 mM E-64 mM, 2mM Leupeptin and 1.5 mM Pepstatin A) (1:100) (Sigma, UK), pH 7.2, 4°C). The samples were thoroughly mixed (20 min) before centrifugation at 10000 g for 10 min. The supernatants were transferred to a fresh tube and centrifuged for a further 10 min period (10000 g). The supernatant was analysed using BD™ Cytometric Bead Array cytokine flex sets (bead based immunoassay - BD Biosciences, USA) for cytokine and chemokine detection (IL6, IL10, IL1β, chemokine (C-X-C motif) ligand 1 (CXCL1) or KC and monocyte chemoattractant protein-1 (MCP-1)). Flow cytometry (Accuri C6 flow cytometer, BD Biosciences, USA) was used to discriminate between different bead populations. The flex sets employ differently sized micro particles with discrete fluorescence intensities to detect soluble analytes.

Anti-oxidant depletion

The mouse liver samples were weighed, thawed on ice and homogenized in 2 ml of lysis buffer (Senft et al. 2000) and incubated for 10 min before being centrifuged at 10000 g for 5 min to generate lysates. Glutathione was quantified in the lysate by reaction of sulfhydryl groups with the fluorescent substrate *o*-phthalaldehyde (Sigma, UK) using a fluorimeter with an excitation wavelength of 350 nm and emission wavelength of 420 nm. The protocol was slightly modified to include measurements of total glutathione by reducing oxidised glutathione dimers (GSSG) by addition of 7 µl of 10 mM sodium dithionite to all samples and incubation at room temperature for 1 hr.

Histology

The liver samples were trimmed, dehydrated and embedded in paraffin on a Tissue-Tek VIP Jr. vacuum infiltration processor (Sakura, The Netherlands). The sections were cut at 5 µm on a Shandon Finesse Microtome (Axlabs, Denmark). The cut sections were stained with haematoxylin and eosin (H&E) before examination by light microscopy (Leica Microsystems, Germany). Three random animals (three slides per animal) were chosen from each group for blinded histological analysis. The slides were randomised before scoring for levels of steatosis, inflammation and necrosis on a 5-class scale.

Statistical analysis

The data is expressed as mean \pm standard error of the mean (SEM). For statistical analysis, the detection of significant differences for all end-points was determined utilising two-way full factorial ANOVA with post-hoc multiple comparisons (Tukey) and a defined significance level of $p < 0.05$. The only exception to the above was the statistical analysis of the *in vitro* cytotoxicity end-point where a full-factorial ANOVA with Tukey's multiple comparison was used. All statistical analysis was carried out via Minitab 17 and Minitab Express.

Results

Particle characterisation

The characterisation data demonstrated that the Ag NPs were well dispersed in both exposure vehicles (PBS and complete cell culture medium containing 10% serum). However, there was a tendency for some agglomeration of the particles in PBS after 24 hr. This was of less importance as the animals were injected with the particles almost immediately following the initial dispersion (< 45 min). In addition, no endotoxin contamination (≤ 0.25 EU/ml) was detected for the Ag NPs.

TABLE 3

In vitro data

Impact of Ag NPs exposure on HepG2 cell cytotoxicity

The analysis of mitochondrial function revealed a concentration-dependent increase in Ag NP-dependent cytotoxicity at 24 hr of exposure (LC_{50} between 4.9 and 9.75 $\mu\text{g}/\text{cm}^2$) (Figure 2). There was no effect of ethanol pre-treatment on hepatocyte cell viability following exposure of the highly cytotoxic Ag NPs. In preliminary experiments, the lactate dehydrogenase (LDH) activity assay was utilised as a means of measuring cell membrane integrity following NP exposure. However, this mode of cytotoxicity assessment was abandoned in the main experiments due to physical interference of the Ag NP with the assay.

FIG 2

Impact of Ag NP exposure on HepG2 IL8 production

The changes in cytokine secretion as a consequence of Ag NP exposure was assessed within the supernatant of non and pre-ethanol treated HepG2 cells. The data demonstrated a clear concentration-dependent increase in levels of chemokine secreted from the hepatocytes up to 19.5 $\mu\text{g}/\text{cm}^2$. The decrease detectable in IL8 levels at the highest concentration could be due to cytotoxicity. Additionally, there was a significant increase in the IL8 secretion from ethanol pre-treated cells compared to the non-pre-treated cells following exposure to the Ag NPs. Finally no ethanol effect was noted in the non-particle treated control groups. This data suggests that ethanol pre-treatment might amplify the *in vitro* inflammatory response following exposure to the Ag NPs.

FIG 3

***In vivo* data**

Bodyweight changes in the alcohol fed and/or Ag NP exposed animals

There was no significant change in the body weight of ethanol vs. control fed and/or Ag NP or AgNO₃ exposed animals (data not shown). In addition, no visible signs of discomfort were noted in any of the mice during the feeding period or following the Ag NP exposure up to 168 hr post exposure (with the exception of the mice exposed to the AgNO₃).

As an important note, some of the AgNO₃ treated animals died 1 day post exposure with the remainder of the animals in these groups sacrificed immediately for ethical reasons. Therefore, no 168 hr data is available for the AgNO₃ for any of the end-points investigated.

Hepatic inflammation

The analysis of hepatic specific inflammation demonstrated significant alterations in the levels of IL10, KC and MCP-1 in liver tissue from mice treated with Ag NPs in a dose-dependent manner (Figure 4). One of the most notable of these changes was observed in terms of the anti-inflammatory IL10 levels – a Ag NP dose-dependent increase in the cytokine levels was observed as well as a significant and large decrease in the levels of IL10 in the alcohol fed mice as compared to the control liquid diet fed animals treated with the NPs (Figure 4a). This is of crucial importance as this data suggests that the overall anti-inflammatory milieu of the healthy liver is disturbed in the chronically alcohol fed animals with these changes largely increased following Ag NP treatment (cumulative alcohol + Ag NP effect of $p < 0.005$). In addition, an Ag NP (high dose) and ethanol induced effect was noted for the two inflammatory chemokines KC (cumulative alcohol + Ag NP effect of $p < 0.005$) and MCP-1 with a significant up-regulation in the protein levels following a 24 hr treatment with the particles. Interestingly, KC returned to background levels after 168 hr irrespective of ethanol feeding. On the other hand, MCP-1 were levels significantly lowered but remained above those observed in the control animals. **There were no significant changes noted in IL6 levels following either alcohol or Ag NP treatment. Finally, hepatic IL1 β levels were below the detection limit of the assay utilised in these experiments for all treatments and time-points investigated.**

FIG 4

Acute phase response

The acute phase response (APR) is a prominent systemic reaction of the organism to local or systemic disturbances in its homeostasis caused by infection, tissue injury, trauma or immunological disorders. **In this experiments plasma SAA3 levels were measured as an indicator of such a response following the NP challenge.** The data showed that the exposure to Ag NPs resulted in a dose dependent increase in SAA3 levels. There was no evidence of an alcohol-mediated response, however, a large and significant incremental

effect was noted in the alcohol fed and Ag NP exposed animals ($p<0.005$). The acute phase response was predominantly resolved at the 168-hr time-point (Figure 5).

FIG 5

Glutathione depletion

As a measure of oxidative stress, the reduced and total glutathione content were measured in the liver homogenates (Figure 5). The data showed a dose dependent decrease in both GSH and total GSH in the Ag NP exposed animals at 24 hr. There was no significant change in glutathione status in the alcohol fed mice. However, an accumulative significant response ($p<0.05$) was noted in the total GSH levels at 24 hr post material exposure. There were no alterations in either the GSH or total GSH quantities in the livers from any of the mice at the latter time-point which advocates that this end-point can only be utilised as a predicator of acute hepatic health.

FIG 6

Blood biomarkers of liver damage

The analysis of blood bio-markers showed some statistically significant changes with regards to ALT, albumin and cholesterol levels. Unfortunately, due to haemolysis in a subset of samples the variations (interference) in the AST readings were larger than otherwise expected. The data demonstrated a significant increase in the ALT levels in the ethanol and NP exposed animals suggesting liver damage. This was accompanied by a decrease in albumin production in the same animals which is a biomarker of liver injury. Interestingly, these responses were not significantly altered in the animals exposed to the alcohol or the Ag NPs independently. In addition, a significant increase in cholesterol concentrations was noted in the blood sourced from animals on the alcohol diet and exposed to the high dose of the Ag NPs at the 168 hr time-

point. This corroborated the above findings advocating liver damage in the specific group of animals. Finally, several time-dependent differences were noted for certain biomarkers investigated, however, no discriminating pattern was observed between these alterations.

TABLE 4

Histology

The histopathological examination of the mice livers revealed that chronic alcohol consumption resulted in significant hepatic injury manifested most notably as steatosis (Figure 7b). These findings were comparable between the animals scarified at 24 and 168 hr (Table 5). The exposure to the Ag NPs resulted in comprehensive pathology in the liver manifested as numerous bi-nucleate hepatocytes, ballooning of hepatocytes, neutrophilic infiltrations, necrotic hepatocytes, areas of necrosis, granuloma formation and the destruction of the liver plates (hepatocyte cords) (Figure 7c-e). In addition to the clearly visible steatosis, similar Ag NP-induced pathological findings were observed (in some instances aggravated) in the alcohol fed and NP exposed animals (Figure 7f-i). The most conspicuous differences between the two groups were noted in the comparisons between the control and alcohol fed animals exposed to the Ag NPs at the 168 hr time-point. In the Ag NP exposed control diet animals clear signs of liver recovery were noted at the later time-point investigated. In contrast the animals in the alcohol diet group exposed to the Ag NPs exhibited similar pathology to their counterparts examined at the 24 hr time-point. It is crucially important to state that there were variations in the degree of pathological damage evident between individuals in each group (images are only representative of the damage observed). However, all the listed pathological findings were evident in all three animals assessed in the appropriate treatment groups. Finally, it should be stated that the damage was not uniform across all regions of the lobes investigated and no discerning pattern was notable.

FIG 7

TABLE 5

Discussion

The accumulation of the NMs in the liver of mice post IV exposure has been established in previous studies (Fernandez-Ruiz et al. 2014; Hirn et al. 2011; Pang et al. 2016). It has been shown that the Ag NPs have adverse effects on a hepatocyte cell line (Gaiser et al. 2013; Kermanizadeh et al. 2012; 2013a), human primary hepatocytes (Kermanizadeh, et al. 2013b) and induce an acute response in the liver *in vivo* in terms of a neutrophil influx and hepatic inflammation (Kermanizadeh et al. 2013c; Kermanizadeh et al. 2014a). In this study, we demonstrate that the IV administration of Ag NPs induced liver specific inflammation, liver anti-oxidant depletion and an acute-phase response. The histopathological examination and analysis of blood bio-markers further corroborated NP-induced liver damage. In this set of trials, we further show that alcohol mediated mild liver damage (as a model for pre-clinical pre-existing medical conditions) is extremely important in terms of orchestrating the overall inflammatory response of the organ before and after Ag NP exposure as well as the resolution and recovery post NP treatment. This study is the first of its kind to investigate NP toxicity in models of alcoholic hepatic disease *in vitro* and *in vivo*, as well highlighting the importance of the inclusion of vulnerable groups (individuals with pre-existing medical conditions) for hazard and risk assessment for nanomaterials.

The analysis of hepatic specific proteins demonstrated significant alterations in the levels of IL10, KC and MCP-1 in liver tissue from mice treated with Ag NPs. One of the most important of these changes was observed in terms of the anti-inflammatory IL10 levels; an Ag NP dose-dependent increase in the cytokine levels was accompanied by a significant and large decrease in the levels of IL10 in the alcohol fed animals as compared to the control liquid diet fed animals treated with the NPs. In previous studies, it has been reported that the IV treatment of mice with a Ag NP with similar physicochemical characteristics also resulted in a large and significant increase in hepatic IL10 production as well as a marked up-regulation of IL10 mRNA (Kermanizadeh et al. 2013c; 2014a). It is hypothesised that the large amounts of IL10 may act as an antagonist against the pro-inflammatory cytokines advocating that tolerance might be favoured over an inflammatory response as a consequence of the NP challenge. It is understood that Kupffer cells are

indispensable in the liver immunity against gut derived antigens and are known to produce a large array of inflammatory cytokines which can further activate hepatic T cells, in turn promoting phagocytosis and cytokine production by Kupffer cells in a positive feedback loop (Bottcher et al. 2011; Seki et al. 2011). However it is believed that Kupffer cells in a non-diseased liver are in a constant semi-activated state and are crucial in maintaining tolerance to food antigens in everyday life (Heymann et al. 2015). Hence the liver offers a unique scenario in which the resident macrophages can both initiate an immune response or play an active role in retaining an immuno-tolerant state (Bottcher et al. 2011; Heymann et al. 2015). In this study the data suggests that the overall anti-inflammatory milieu of the healthy liver is disturbed in the chronically alcohol fed animals with these changes being augmented following NP treatment making the organ vulnerable to uncontrolled inflammatory response and damage.

In association to the above, we have previously shown that any changes in the inflammatory status of the liver following NP treatment had returned to background levels by 72 hr post exposure (Kermanizadeh et al. 2013c; 2014a). In this study this was not the case in the NP-treated alcohol fed animals in which the IL10 levels and MCP-1 levels remained significantly different from the controls for at least up to 168 hr after the NP exposure.

The analysis and summary of the blood biochemistry of biomarkers in alcohol and Ag NP exposed mice showed significant liver damage which was intensified in the Ag NP and alcohol exposed animals. It has been previously reported that impaired lipid metabolism is often associated with liver disease (Chrostek et al. 2014). To the best of our knowledge no other studies have investigated blood biochemistry following IV exposure to Ag NPs. IV exposure to 10 and 60 nm PEG gold NPs - 4 mg per animal over 28 days resulted in changes in blood biochemistry indicative of liver damage (Zhang et al. 2011). As an important side note it should be stated that previous studies have demonstrated that chronic ethanol diets result in mild changes in blood bio-markers of liver toxicity which are similar to the findings in this study (Bertola et al. 2013; Cohen et al. 2010; Mandrekar et al. 2011).

As a measure of oxidative stress, the reduced and total glutathione content were measured in liver homogenates. The data demonstrated a dose-dependent decrease in glutathione levels compared to vehicle control animals after exposure to the Ag NPs 24 hr post exposure. However, there was no significant change in anti-oxidant status in the alcohol fed animals. This is in contradiction to previous studies in which it has been suggested that excessive uptake of alcohol causes a production of free radicals and a decrease of GSH levels albeit these measurements are often made in serum (Ojeda et al. 2016; Tian et al. 2016; Wang et al. 2016). Hepatocytes contain about 10% of the total human body pool of GSH (Loguercio et al. 2003), therefore, it is not unconceivable that chronic alcohol feeding alone in this study is not sufficient to induce an anti-oxidant depletion in the liver homogenates. Another potential explanation for the observed data is the fact that the maintenance of the redox balance is extremely complex and constantly changing hence it is very possible that these time-points are not ideal for peak measurements for alcohol-induced GSH depletion. Conversely, a Ag NP and alcohol induced accumulative and augmented decrease in GSH levels was observed at 24 hr post material exposure. Finally, no variation in the reduced GSH or total GSH quantities in the liver homogenates were noted for any of the animals at the latter time-point. In a previous study of anti-oxidant status in the liver, IV administration of 50 µg of Ag NPs (20 nm) per rat had no significant effect in hepatic GSH levels 24 hr after exposure (Gaiser et al. 2013). The disparities in the observed data presented here and the Gaiser study can be explained by the differences in the dose and species of animals utilised. However, the intratracheal instillation of a 20 nm Ag NP resulted in a dose-dependent decrease in GSH levels in the liver of exposed mice (Gosens et al. 2015). Additionally, similar depletion in GSH levels has been reported after Ag NP exposure of C3A hepatocytes (Kermanizadeh et al. 2012) and human Chang liver cells (Piao et al. 2011).

Acute phase proteins are a range of blood proteins primarily synthesized by **hepatocytes in the liver, lung or adipocytes** (Han et al. 2007; Saber et al. 2014) as part of the APR. The APR is part of the innate immune system, which is triggered by different stress stimuli including trauma, infection or inflammation. The APR is a core part of the innate immune response and is observed across all animal species. To date a total of around 200 acute phase proteins have been identified (Eklund et al. 2012). The biological activities of these

proteins are vast and have been extensively described previously (Cray *et al.* 2009). The plasma SAA concentration begins to increase 3-6 hr after an inflammatory stimulus, peaks on day 3, and returns to baseline levels after day 4. It is widely understood that SAA is highly immunologically active and possess many pro-inflammatory properties. In previous rodent experimental models SAA3 has been shown to recruit macrophages and neutrophils (Badolato *et al.* 1994; Saber *et al.* 2013) as well as inducing the development of a Th17 response (Ather *et al.* 2011). In our experiments the exposure to Ag NPs resulted in a dose-dependent increase in SAA3 levels. There was no evidence of an alcohol-mediated response however a large and significant incremental effect in the levels of serum SAA3 was noted in the alcohol fed and Ag NP exposed mice. To the best of our knowledge no studies have investigated the acute phase response following exposure to Ag NPs in any *in vivo* settings. However, the *in vitro* exposure of C3A cells to Ag NPs (~ 20 nm) at concentrations of up to 40 µg/cm² did not result in the production of C-reactive protein from the hepatocytes (Kermanizadeh *et al.* 2013a). In accordance to findings in this study, the intratracheal exposure of C57BL/6 mice to short (~ 1 µm) and long (~ 4 µm) multi-walled carbon nanotubes at doses of 54 and 162 µg per animal also resulted in significantly increased SAA3 levels in the serum of the exposed animals (Poulsen *et al.* 2015).

The histological examination of the mice livers revealed that the alcohol consumption resulted in steatosis. These findings were comparable between the animals scarified at 24 and 168 hr. Similar findings have been reported previously in alcohol fed rodents (Anthony *et al.* 2010; Escher *et al.* 2006; Ki *et al.* 2010; Nguyen *et al.* 2007). The exposure to the Ag NPs resulted in comprehensive and severe pathology in the liver manifested as inflammatory, degenerative and necrotic changes. The degenerative changes in the Ag NP treated groups were observed in all three zones of the hepatic lobules. Additionally, there were some instances of increased infiltration of inflammatory cells in the Ag NP exposed alcohol fed animals in comparison to the same NP treatment group on the control diet. Interestingly and importantly, the amount of NP-induced damage in the animals on the alcohol diet was considerably more notable and visible at 168 hr post exposure in comparison to the Ag NP exposed animals on the control diet at the same time point. These observations clearly demonstrate that the liver's capacity for self-regeneration and recovery is compromised

due to alcohol consumption. In our previous investigations, we have shown that any hepatic adverse effects following the IV exposure to a 20 nm Ag NP had resolved by 78 hr post exposure (Kermanizadeh et al. 2013c; 2014a). These crucial differences perceived in this study compared to previous findings once again emphasizes the importance of the inclusion of persons or groups with pre-existing medical complications in any nanomaterial hazard or risk assessment strategies. Previous investigations into Ag NP toxicity and bio-distribution following treatment with different Ag NPs/doses/routes of exposure have also revealed varying degrees of similar hepatic histological pathologies (i.e. Ansari et al. 2016; Guo et al. 2016; Patlolla et al. 2015; Recordati et al. 2016).

Ag NPs dispersed in any aqueous medium will release a degree of soluble Ag ions. Therefore, it is necessary to distinguish between the toxic effects of Ag NPs and the dissolved Ag ion content. This is important as it has been demonstrated that under certain conditions a significantly larger proportion of Ag ions are translocated to the liver in comparison to Ag NPs (van der Zande et al. 2012). In this study, to evaluate the role of Ag ions in the effects observed above, certain groups of mice were exposed to a dose of AgNO₃ which was equivalent to 100% dissolution of the Ag NPs. Unfortunately, the high dose of AgNO₃ administered intravenously proved to be very toxic hence a direct comparison of Ag NP vs. ionic effects is difficult. This being said a number of differences were noted between the exposure groups (24 hr) which suggests that some of the hepatic adverse effects described could at least partially be accredited to the NPs (i.e. acute phase response) while others were more likely to be principally mediated by the Ag ions (i.e. IL6 production). Although it is impossible to state unequivocally it appears that not all the adverse effects observed in this study are attributable to the Ag ions.

To corroborate our *in vivo* findings, the effects of Ag NP exposure on cell viability and inflammation in ethanol pre-treated hepatocytes (HepG2) was investigated *in vitro*. The data showed that alcohol pre-treatment had no effect on cell viability *in vitro*, however, a significant increase in IL8 secretion was detected in ethanol pre-treated cells compared to the non-ethanol treated hepatocytes. These observations suggest that ethanol pre-treatment might amplify the hepatocyte inflammatory response following exposure to the Ag

NPs. This data complements the inflammatory data generated in the *in vivo* experiments described above. To date no other *in vitro* study has investigated NP-induced adverse health effects in ethanol pre-treated hepatocytes, however Ag NP mediated high cytotoxicity and IL8 production has been previously reported for hepatocyte cell lines (Kermanizadeh et al. 2013a), human primary hepatocytes (Kermanizadeh et al. 2013b) and 3D human liver micro-tissue (Kermanizadeh et al. 2014b). The suitability of hepatocyte cell lines as a representable model for predicting *in vivo* hepatic alcohol and NP mediated effects require further investigation. Future studies utilising a wide range of appropriate end-points and assorted panel of nanomaterials would shed further light on the suitability of the use of hepatic cell lines as predictors of *in vivo* effects in this context.

Despite the many insights offered in this study, there are a few limitations that need to be considered for future experimentations. The most widely used model for alcoholic liver injury involves chronic feeding with the Lieber-DeCarli liquid diet containing ethanol for 4-6 weeks. However, the National Institute on Alcohol Abuse and Alcoholism (NIAAA) model (chronic alcohol feeding plus binge alcohol feeding model in mice) (Bertola et al. 2013) mimics the drinking pattern in alcoholic hepatitis patients. The NIAAA model may also be beneficial for the investigation of alcohol and NP related damage to other organs, such as the pancreas, spleen, kidneys and the lungs. Future studies should consider the use of this altered alcohol feeding protocol for more realistic representation of chronic alcohol damage. In any toxicological study, it is imperative that great attention is paid to the selection of biologically relevant doses for the investigation of bio-kinetics and toxicological end-points. It should be clearly stated that the 100 µg NP dose utilised in this study is rather high if not necessarily physiologically irrelevant. As previously touched upon this is the first study investigating alcohol and NP mediated health effects concomitantly, therefore, it was important to have at least one high dose to allow for the differentiation of NP effects. Due to the financial and ethical restraints, we were unable to incorporate more than two doses in the current study. The utilisation of low and repeated exposures will be highly advantageous in the future. Any upcoming studies will also investigate a wider range of time-points post material exposure to identify the optimum (peak) epochs for certain end-points (acute phase response, cytokine production - i.e. the peak time point for the detection of IL1 β and IL6 could

have been much earlier than 24 hr which was utilised in our experiments. This could explain the increased SAA3 levels and the low levels of IL1 β and IL6 detected in this study). It would be very interesting to assess NP induced adverse effects following inhalation and oral exposure routes in the hepatic disease model.

In this study the adverse health effects of Ag NP exposure were investigated in models of alcoholic hepatic disease *in vitro* and *in vivo*. The data showed that NP induced hepatic health effects were aggravated in the alcohol pre-treated mice in comparison to controls with regards to an organ specific inflammatory response, changes in blood biochemistry, the acute phase response and pathology. In addition, alcoholic disease influenced the organ's ability for recovery post NP challenge. These important findings require serious consideration in future risk and hazard assessment approaches for engineered nanomaterials.

Conflict of interest

The authors declare no conflicts of interest.

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Figure legends

Figure 1. The diagrammatic representation of the feeding schedule

Figure 2. Cytotoxicity in control and ethanol pre-treated HepG2 cells following the exposure to the Ag NPs. The cells were exposed to cell medium (control - C) or increasing concentrations of the Ag NPs for 24 hr with cell viability measured via WST-1 assay. The values represent mean \pm SEM (n=3), significance between the groups indicated by ** = p<0.005 (full-factorial ANOVA statistical analysis)

Figure 3. IL8 production within HepG2 cell supernatants following exposure to increasing concentrations of the Ag NPs for 24 hr. The values depict mean \pm SEM (n=3), significance indicated by * = p<0.05 and ** = p<0.005 when indicative of a NP-induced effect and ϕ = p<0.05 and $\phi\phi$ = p<0.005 when an alcohol related effect is exhibited (two-way full factorial ANOVA statistical analysis)

Figure 4. Cytokine secretion from control and alcohol fed mice livers following IV exposure of 25 or 100 μ g of Ag NPs for 24 or 168 hr. The values depict mean \pm SEM (n=5), significance indicated by * = p<0.05 and ** = p<0.005, when indicative of an alcohol induced effect, & = p<0.05 and && = p<0.005, when indicative of an Ag NP induced effect and α = p<0.05 and $\alpha\alpha$ = p<0.005 when a time-dependent effect is exhibited (two-way full factorial ANOVA statistical analysis)

Figure 5. SAA3 measured in the serum of control and ethanol fed mice exposed to 25 (low) and 100 (high) μ g of the Ag NPs for 24 or 168 hr. The values depict mean \pm SEM (n=5), significance indicated by * = p<0.05 and ** = p<0.001, when indicative of a NP-induced effect and $\alpha\alpha$ = p<0.005 of alcohol related effect (two-way full factorial ANOVA statistical analysis)

Figure 6. Reduced GSH (GSH) and total glutathione (Total GSH) measured in the livers of control and ethanol fed mice exposed to 25 and 100 μ g of the Ag NPs for 24 or 168 hr. The values depict mean \pm SEM (n=5), significance indicated by * = p<0.05 and ** = p<0.005, when indicative of a NP-induced effect (two-way full factorial ANOVA statistical analysis)

Figure 7. The histopathological examination of H&E stained liver tissue from **a)** mice on control diet, **b)** mice on the alcohol supplemented diet - damage most evident by steatosis, fatty changes and vacuolar degeneration, **c-e)** control diet mice exposed to 100 μ g of Ag NPs for 24 hr - liver inflammation, dramatic changes in intrahepatic architecture, granuloma formation in different zones of the liver, hepatocyte necrosis, parenchymatous degeneration, vacuolar generation and the destruction of the liver plates **f-i)** mice on alcohol supplemented diet exposed to 100 μ g of Ag NPs for a period of 24 hr. Black arrows - bi-nucleate arrows, purple arrows - ballooning of hepatocytes, red arrows - steatosis, brown boxes - inflammatory cell influx, blue boxes - necrotic hepatocytes, orange boxes - granuloma formation, green boxes - destruction of liver plates. Scale bar - 25 μ m

Supplementary Figure legend

Supplementary figure 1. Low magnification (10x) histopathological examination of H&E stained liver tissue from **a)** mice on control diet, **b)** mice on the alcohol supplemented diet, **c)** control diet mice exposed to 100 μ g of Ag NPs for 24, **d)** mice on alcohol supplemented diet exposed to 100 μ g of Ag NPs for a period of 24 hr

Tables

Table 1 The nutritional profile of the two Lieber-Decarli 82 diets utilised in the feeding schedule (Kcal/litre)

Component	Control diet	Ethanol diet
Protein	151	151
Fat	359	359
Carbohydrate	490	135
Ethanol + maltose dextrin	0	355

Table 2 The outline of the feeding and experimental treatment groups

Diet	Treatment	Time point	# of animals
Control	PBS	24 hr	5
Ethanol	PBS	24 hr	5
Control	low dose Ag NP	24 hr	5
Ethanol	low dose Ag NP	24 hr	5
Control	high dose Ag NP	24 hr	5
Ethanol	high dose Ag NP	24 hr	5
Control	AgNO ₃	24 hr	5
Ethanol	AgNO ₃	24 hr	5
Control	PBS	168 hr	5
Ethanol	PBS	168 hr	5
Control	low dose Ag NP	168 hr	5
Ethanol	low dose Ag NP	168 hr	5
Control	high dose Ag NP	168 hr	5
Ethanol	high dose Ag NP	168 hr	5
Control	AgNO ₃	168 hr	5
Ethanol	AgNO ₃	168 hr	5

Table 3 The average mode and mean hydrodynamic size of the Ag NPs dispersed in PBS and hepatocyte complete medium

	Mean (nm) - 30 mins	Mode (nm) - 30 mins	Mean (nm) - 24 hr	Mode (nm) - 24 hr
PBS	27.2±0.0	27.1±0.1	174.1±15.6	166.0±23.7
Complete MEM	42.5±8.5	33.8±5.8	36.2±1.5	38.2±0.2

Mode - the size most abundant in the measurements

Mean - the average of the size in the measurements

Table 4 Blood bio-markers of liver toxicity assessed in the serum of Ag NP exposed animals sacrificed 24/168 hr post treatment

Biomarker	Control diet - PBS	Ethanol diet - PBS	Control diet - low Ag	Ethanol diet - low Ag	Control diet - high Ag	Ethanol diet - high Ag
ALT (U/l) - 24 hr \diamond	23.5 \pm 1.7	30 \pm 2.9	27.4 \pm 1.0	24.0 \pm 1.7	28.3 \pm 2.1 \square	45.8 \pm 7.5* α
ALT (U/l) - 168 hr	30.3 \pm 2.7	36.0 \pm 4.8	31.2 \pm 4.0	37.8 \pm 4.3	31.2 \pm 3.0	34.4 \pm 3.6
AST (U/l) - 24 hr	90.8 \pm 15.1 \square	203.7 \pm 24.6	147.9 \pm 6.9	131.7 \pm 19.5 \square	128.5 \pm 7.6 \square	271.6 \pm 64.3
AST (U/l) - 168 hr	206.4 \pm 13.5 \square ϕ	178.6 \pm 54.4	239.7 \pm 34.8 \square ϕ	157.4 \pm 14.7	390.4 \pm 85.8 \square ϕ	311.3 \pm 63.8 \square
Albumin (g/l) - 24 hr	12.4 \pm 0.01	13.3 \pm 0.3*	12.6 \pm 0.2	12.5 \pm 0.1	11.9 \pm 0.1	12.5 \pm 0.3
Albumin (g/l) - 168 hr	13.6 \pm 0.2 $\phi\phi$	13.3 \pm 0.3	12.5 \pm 0.3 α	12.12 \pm 0.2 α	12.9 \pm 0.3 ϕ	11.6 \pm 0.2* $\alpha\alpha\phi$
Cholesterol (mmol/l) - 24 hr	2.1 \pm 0.1	2.1 \pm 0.02	2.0 \pm 0.1	2.0 \pm 0.1*	2.2 \pm 0.1	2.2 \pm 0.6
Cholesterol (mmol/l) - 168 hr $\diamond\diamond$	2.0 \pm 0.03	2.1 \pm 0.1	1.9 \pm 0.0	2.2 \pm 0.0	1.9 \pm 0.02	2.4 \pm 0.1 $\alpha\alpha$ **
Triglycerides (mmol/l) - 24 hr	0.8 \pm 0.1	0.9 \pm 0.2	0.6 \pm 0.1	0.7 \pm 0.1	0.6 \pm 0.1	0.5 \pm 0.04
Triglycerides (mmol/l) - 168 hr	0.5 \pm 0.03 $\phi\phi$	0.6 \pm 0.1 $\phi\phi$	0.4 \pm 0.03 $\phi\phi$	0.5 \pm 0.04 $\phi\phi$	0.3 \pm 0.03 $\phi\phi$	0.5 \pm 0.1

The values depict mean \pm SEM (n=5), ALT (alanine aminotransferase U/l), AST (aspartate aminotransferase U/l), albumin (g/l), cholesterol (mmol/l), triglycerides (mmol/l) with significance indicated by * = p<0.05 and ** = p<0.005, when indicative of an alcohol induced effect, α = p<0.05 and $\alpha\alpha$ = p<0.005 when a NP-induced effect is observed and ϕ = p<0.05 and $\phi\phi$ = p<0.005 when a time-dependent effect is exhibited. Interaction of alcohol and NP effects signified by \diamond = p<0.05 and $\diamond\diamond$ = p<0.005. \square = outlier value removed (two-way full factorial ANOVA statistical analysis)

Table 5 The histological score of liver pathology from three slides from three random animals for each treatment group ranked from 0-5

24 hr	Control diet - PBS	Ethanol diet - PBS	Control diet - low Ag	Ethanol diet - low Ag	Control diet - high Ag	Ethanol diet - high Ag
Steotosis	0, 0, 0	4, 4, 3	0, 0, 0	3, 3, 4	0, 0, 1	4, 5, 3
Inflammation	0, 0, 0	0, 0, 1	1, 1, 1	2, 2, 1	5, 5, 4	4, 5, 4
Necrosis	0, 0, 0	0, 0, 0	0, 0, 0	1, 0, 1	4, 3, 4	5, 4, 4

168 hr	Control diet - PBS	Ethanol diet - PBS	Control diet - low Ag	Ethanol diet - low Ag	Control diet - high Ag	Ethanol diet - high Ag
Steotosis	0, 0, 0	5, 5, 5	0, 1, 0	5, 5, 4	0, 0, 0	5, 5, 5
Inflammation	0, 0, 0	0, 1, 0	1, 0, 1	1, 1, 1	2, 1, 1	4, 4, 4
Necrosis	0, 0, 0	0, 0, 0	0, 0, 0	1, 2, 2	1, 0, 1	4, 3, 5